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16. ABSTRACT

The DNA adducts formed in Salmonella typhimurium when bacteria are incubated with radioactive benzo(a)pyrene and liver microsomal enzymes from several sources has been investigated. When enzyme prepartions from Aroclor 1254 or 3-methylcholanthrene induced C57BL/6N (B6) mice wereused to mediate activation, the predominant product was n adduct between the 10 position of 7B,8a-dihydroxy-9a,10a epoxy-7,8,9,10-tetra-hydrobenzo(a)pyrene and the N-2 position of deoxyguanosine. Similar results were obtained with human liver and with Aroclor-induced rat-liver enzyme preparations. This adduct is also the major DNA product previously found when human tissues or certain rodent cells were inducbated with benzo(a)pyrene. On the other hand, when activation of benzo(a)-pyrene was mediated by a phenobarbital-induced B6 mouse-liver enzyme preparation, the extent of binding was quite low and the profile of DNA adducts in S. typhimurium DNA was quite different. Thus, under appropriate conditions, the activation and DNA binding of benzo(a)-pyrene in the microsome mediated S. typhimurium mutagenesis assay generally resembles that seen in intact mammalian cells. Caution must be exercised, however, in the choice of microsome-activation systems.

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DNA—BENZO[*a*]PYRENE ADDUCTS FORMED IN A *Salmonella typhimurium* MUTAGENESIS ASSAY SYSTEM

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Summary

The DNA adducts formed in *Salmonella typhimurium* when bacteria are incubated with radioactive benzo[*a*]pyrene and liver microsomal enzymes from several sources has been investigated. When enzyme preparations from Aroclor 1254 or 3-methylcholanthrene induced C57BL/6N (B6) mice were used to mediate activation, the predominant product was an adduct between the 10 position of 7β,8α-dihydroxy-9α,10α epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene and the N-2 position of deoxyguanosine. Similar results were obtained with human liver and with Aroclor-induced rat-liver enzyme preparations. This adduct is also the major DNA product previously found when human tissues or certain rodent cells were incubated with benzo[*a*]pyrene. On the other hand, when activation of benzo[*a*]pyrene was mediated by a phenobarbital-induced B6 mouse-liver enzyme preparation, the extent of binding was quite low and the profile of DNA adducts in *S. typhimurium* DNA was quite different. Thus, under appropriate conditions, the activation and DNA binding of benzo[*a*]pyrene in the microsome mediated *S. typhimurium* mutagenesis assay generally resembles that seen in intact mammalian cells. Caution must be exercised, however, in the choice of microsome-activation systems.

Short-term, or in vitro tests for monitoring environmental agents for mutagenicity and carcinogenicity have increased in use dramatically. In particular,

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the *Salmonella typhimurium* assay developed by Ames et al. [1] is being used to predict the carcinogenic potential of many compounds. This assay incorporates the use of a microsomal enzyme preparation to mimic the in vivo metabolic activation that is necessary for expression of the mutagenic and carcinogenic potential of many compounds.

If this assay is to be used as a valid predictor of the response of humans to carcinogens, it is essential to determine whether these compounds are metabolized and bound to cellular DNA in this assay in a similar manner as in mammalian cells and human tissues.

To study this problem, we have chosen benzo[*a*]pyrene (BP) since detailed information is available on its metabolism and binding to nucleic acids in a variety of mammalian systems. We have investigated the nature of the BP nucleoside adducts formed in the DNA of the *S. typhimurium* cells in this assay system, using liver enzymes from different species and induced by various compounds.

Materials and methods

Reagents. NADP, glucose 6-phosphate and alkaline phosphatase (*E. coli* type III) were purchased from Sigma Chemical Co. St. Louis, MO; venom phosphodiesterase, spleen phosphodiesterase, and deoxyribonuclease I were from Worthington Biochemical, Freehold, NJ; and *N. crassa* endonuclease was from Miles, Inc., Elkhart, IN. Generally tritiated [^{3}H]BP (20 Ci/mmol) was obtained from Amersham Corp., Arlington Heights, IL; 4-(*p*-nitrobenzyl) pyridine and 3-methylcholanthrene (3MC) was from Aldrich Chemical Co., Milwaukee, WI; Columbia broth came from Becton, Dickinson and Co., Cockeysville, MD; Aroclor 1254 was from Monsanto Co., St. Louis, MO.

Bacteria. Salmonella typhimurium strain TA98 was obtained from Dr. Bruce Ames, University of California, Berkeley, CA.

Animals. Sprague-Dawley rats were supplied by the Animal Facilities, Cancer Center/Institute of Cancer Research, Columbia University College of Physicians and Surgeons, NY, and C57BL/6N mice were obtained from the Charles River Breeding Labs, Inc., Wilmington, MA. The 3MC treatment consisted of a single intraperitoneal dose (80 mg/kg) in corn oil 48 prior to killing. Phenobarbital treatment consisted of two consecutive daily injections of 80 mg/kg. Animals were killed on the 3rd day. Aroclor treatments consisted of a single intraperitoneal dose (500 mg/kg) 5 days before killing.

Experimental procedures

Enzyme preparation. S9 mix was prepared from induced animals by the procedure of Ames et al. [1]. A sample of human S9 was obtained from Dr. Bruce Ames, University of California, Berkeley, CA. Protein was assayed by the method of Lowry et al. [14].

Binding of [^{3}H]BP to Salmonella typhimurium. Overnight cultures of *S. typhimurium* strain TA98 were grown in 100 ml of Columbia broth, centrifuged, and resuspended in 3 ml of a cofactor mix containing 8 μmol $MgCl_2$, 33 μmol KCl, 5 μmol glucose 6-phosphate, 4 μmol NADP and 100 μmol sodium

phosphate (pH 7.4) per ml. The final concentration of bacteria was 3 to 5 × 10^{10} cells per ml. S9 (8 mg protein) and 0.1 μmol [^{3}H]BP in 50 μl DMSO were added and the samples incubated at 37° C in the dark with shaking for 1 h. The modified DNA was isolated by the procedure of Marmur [15]. After phenol extraction and RNAase digestion, the DNA was extensively washed with ethyl acetate and ether until no radioactivity remained in the wash. The DNA was denatured and enzymatically digested to deoxynucleosides as previously reported [21].

Toluene treatment and sonication of bacteria. Overnight cultures of *S. typhimurium* after centrifugation and resuspension in cofactor mix as above, were treated with 1% toluene. Sonication of the cells was performed with one 15-sec burst at 6 micron on a MSE Sonicator.

Chromatography. Enzymatically hydrolyzed DNA samples were chromatographed on a 65 cm × 1.5 cm column of Sephadex LH-20 using a gradient of 400 ml 30% methanol in water and 400 ml of absolute methanol. A UV marker, 4-(*p*-nitrobenzyl)pyridine, was added to the samples.

Further characterization of the BP—nucleoside products was performed by high-pressure liquid chromatography (HPLC) on a DuPont 830 instrument as described [8] using Waters μ Bondapak C_{18} columns eluted with a 30—60% methanol—water gradient at 50° C.

A UV marker for BP—nucleoside adducts was prepared by reacting (±)-7β, 8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE I) (Chemical Repository, National Cancer Institute, Bethesda, MD) with native calf-thymus DNA as previously reported [9]. This modified DNA was digested enzymatically and the hydrophobic deoxynucleoside adduct isolated and purified by Sephadex LH-20 and HPLC chromatography, using the same procedures as described above for the bacterial DNA.

Results

Liver S9 fractions were prepared from C57BL/6N (B6) mice induced with Aroclor, 3-methylcholanthrene or phenobarbital. Concentrated cultures of *S. typhimurium* TA98 were incubated with [^{3}H]BP, the different S9 preparations, and cofactors in a liquid suspension. This liquid incubation is similar to the preincubation system used by others [20] and was used in order to obtain sufficient quantities of bacterial DNA for isolation of adducts. Measurement of the mutagenicity of BP either by incorporation of bacteria, BP and enzymes directly on the agar plate or by the liquid-preincubation system gave identical results.

Following isolation of the bacterial DNA as outlined in Materials and Methods, the specific activity was determined. Table 1 shows the binding ratio of BP per nucleoside for the different enzyme preparations. The values are in the range of one BP per 10^8 or 10^9 nucleosides; the highest binding was found with Aroclor-induced and the lowest with phenobarbital-induced enzymes. Even the highest value is, however, several orders of magnitude lower than that seen when naked calf-thymus DNA is incubated with [^{3}H]BP and S9 enzymes (about 3 BP per 10^5 nucleosides) [18] or when [^{3}H]BP is incubated with mammalian cell-culture systems (about one BP per 10^5 nucleosides) [7]. Be-

TABLE 1

EFFECTS OF ENZYME SOURCES ON EXTENT OF MODIFICATION OF *S. typhimurium* DNA

Species	Inducer	Extent of DNA modification [a] [^{3}H]BP/10^8 nucleosides
Mouse, C57BL/6N	Aroclor 1254	8.1
	Phenobarbital	0.60
	3-Methylcholanthrene	1.1
Rat, Sprague-Dawley	Aroclor 1254	1.0
Human		0.21

[a] Residues of [^{3}H]BP bound to DNA per 10^8 nucleoside residues. Intact *S. typhimurium* were incubated with [^{3}H]BP, cofactors and the indicated S9 enzyme preparation. DNA was isolated, extensively extracted to remove non-covalently bound material and its specific activity determined. For details see Materials and Methods.

cause of the low extent of binding obtained with *S. typhimurium*, an attempt was made to determine whether increasing the permeability of the bacterial cells would increase the extent of [^{3}H]BP binding to DNA. When the bacteria were first exposed to 1% toluene, or sonicated, prior to incubation with [^{3}H]BP and Aroclor mouse-liver S9, the specific activity of the subsequently isolated bacterial DNA was 3 and 9 times, respectively, higher than that obtained with intact bacteria. Thus increasing the permeability of the cells did

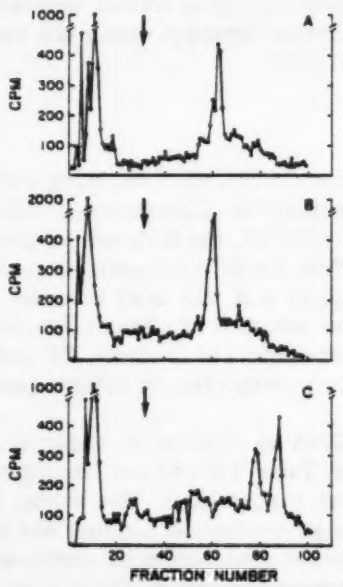

Fig. 1. Sephadex LH-20 column chromatography of enzymatic digests of *S. typhimurium* DNA isolated from bacteria incubated with [^{3}H]BP and the S9 fraction from C57BL/6N mouse liver. Enzyme induction with: (A) Aroclor 1254, (B) 3-methylcholanthrene, (C) phenobarbital. The column was eluted with a 30—100% methanol gradient in water. The arrow indicates the position of the ultraviolet marker 4-(*p*-nitrobenzyl)pyridine.

increase the extent of binding. The binding still did not approach, however, the level seen in mammalian cells.

To determine the types of BP-DNA adducts formed in *S. typhimurium*, the modified DNA was degraded to nucleosides and chromatographed on Sephadex LH-20.

Fig. 1 shows a comparison of the LH-20 column radioactivity profiles of DNA digests obtained from bacteria incubated with [^{3}H]BP and various S9 mouse-liver enzyme preparations. In all samples, a considerable portion (up to 60%) of the radioactivity eluted in the early fractions (fractions 5—20). The remaining material, more characteristic of BP—nucleoside adducts [17], was retained by the column and eluted only with higher concentrations of methanol. The occurrence of the more hydrophilic early eluting material was not restricted to the *S. typhimurium* system. When calf-thymus DNA was incubated with [^{3}H]BP and Aroclor-induced mouse-liver S9 enzymes, enzymatic digests of the modified DNA gave a profile rather similar to that seen in Fig. 1A. Nor is it due to the fact that BPDE-modified DNA is not completely digested enzymatically. When calf-thymus DNA was modified non-enzymatically with [^{3}H]BPDE I and digested enzymatically, all of the radioactivity was tightly retained by the LH-20 column and eluted in the region of fraction 60 of the methanol gradient. In addition, when the radioactive material present in fractions 5—20 of Fig. 1A was collected and resubjected to enzymatic hydrolysis or treated with 0.1 M HCl, the majority of the radioactivity still eluted in the early region of the LH-20 column. This early eluting material in digests of [^{3}H]BP-modified DNA has been previously noted in other in vitro studies [4,11,17,18].

With the Aroclor- and 3MC-induced mouse-liver S9 preparations, the *S. typhimurium* DNA contained a major radioactive adduct which eluted in the

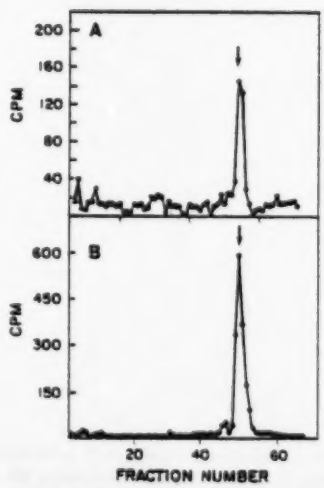

Fig. 2. HPLC profiles of the material that eluted in the region of fraction 61 of the LH-20 columns shown in Fig. 1A and 1B. Aroclor 1254 (A), and 3-methylcholanthrene (B) induced S9 preparations. The arrow indicates the position of the uv marker, BPDE I—deoxyguanosine, N^2(10S-(7R,8S,9R-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene]yl)-deoxyguanosine.

region of fraction 61 of the LH-20 column. This material was collected and further characterized by HPLC. In both cases, the radioactivity eluted as a single homogeneous peak whose elution position was identical to that of an in vitro marker synthesized by reacting BPDE I with DNA followed by enzymatic hydrolysis to nucleosides (Fig. 2). Previous studies have elucidated the structure of the latter product. It is the result of covalent linkage between the C-10 of BPDE I and the N^2 of deoxyguanosine [8].

The use of phenobarbital-induced mouse-liver S9 in the *S. typhimurium* system resulted in different [^{3}H]BP—DNA adducts than those obtained with the Aroclor or 3MC S9 preparation. Fig. 1C indicates that with the former material, the LH-20 profile contained several radioactive materials with two distinct peaks at fractions 78 and 87, and only a questionable peak at fraction 61. Somewhat similar profiles have been found by Boobis et al. [4] with digests of salmon-sperm DNA incubated with phenobarbital-induced B6 mouse-liver S9 enzymes and [^{3}H]BP. Their studies suggest that the peaks at 78 and 87 correspond to nucleoside adducts formed from the binding of benzo[*a*]pyrene 4,5-oxide and 9-hydroxybenzo[*a*]pyrene 4,5-oxide, respectively. Because of the complex profile and the limited amount of these materials, we have not characterized our material further.

To determine the species specificity of the S9 enzyme with respect to the [^{3}H]BP—nucleoside adducts formed in *S. typhimurium*, further experiments were carried out with an S9 fraction prepared from Aroclor-induced Sprague-Dawley rat liver and with an S9 fraction from human liver (autopsy material). The DNA-binding ratio obtained with Aroclor-induced rat-liver enzymes was

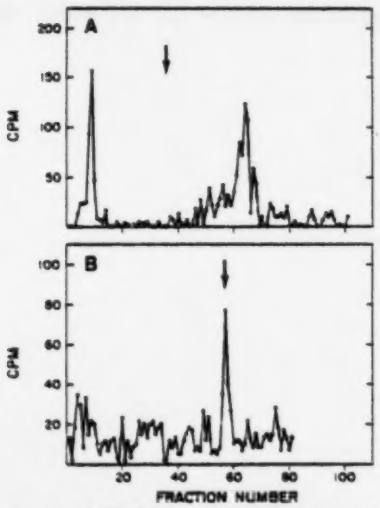

Fig. 3A. Sephadex LH-20 column chromatography of an enzymatic digest of *S. typhimurium* DNA isolated from bacteria incubated with [^{3}H]BP and human liver S9. The arrow indicates the position of the UV marker 4-(*p*-nitrobenzyl)pyridine Fig. 3B. HPLC profile of the material that eluted in the region of fraction 64 of the LH-20 column shown in Fig. 3A. The arrow indicates the position of the UV marker. BPDE I—deoxyguanosine adduct. N^2(10S-[7R,8S,9R-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene]yl)-deoxyguanosine.

1.0 BP residue/10^9 bases, which is about 1/8th that obtained with the S9 preparation from Aroclor-induced B6 mice (Table 1). With the human-liver enzyme preparation the ratio was even lower: 0.21 BP residues/10^9 bases (Table 1). Sephadex LH-20 column chromatography of an enzymatic digest of the latter material revealed a prominent peak in the region of fraction 61 (Fig. 3A) which when rechromatographed on HPLC has the same elution position as the above described BPDE I—deoxyguanosine adduct (Fig. 3B). Similar results were found with digests of the material obtained with rat-liver S9 (data not shown). The radioactive component which elutes early in the LH-20 column, seen in the above studies with mouse-liver enzyme (Fig. 1), was also seen in the studies with human-liver or rat-liver S9 preparations.

Discussion

The present results show that the type of inducer administered to the animal in the preparation of the S9 fraction influences the nature of the [^{3}H]BP—DNA adducts formed in the microsome-mediated *S. typhimurium* mutagenesis-assay system. With Aroclor-induced S9 enzymes from rat and mouse liver, 3MC induced S9 enzymes from mouse, or human liver S9 enzymes, the major [^{3}H]BP—nucleoside adduct that is present in the *S. typhimurium* DNA results from the formation of a covalent adduct between position 10 of the BP metabolite BPDE isomer I and the N-2 position of deoxyguanosine. It is of interest that this adduct is also the major one detected in DNA when human or bovine bronchial explants [8] or human colon [2] are exposed to [^{3}H]BP. It is also a major BP—DNA adduct when mouse-skin [13] or rodent-cell cultures are incubated with radioactive BP [7,5]. Lesser amounts of BPDE isomer II adducts and adducts between BPDE and deoxyadenosine and deoxycytosine [7,16] have also been detected in mammalian systems and it is possible that some of the minor peaks seen in Fig. 1A, 1B and 3A represent these materials in *S. typhimurium* DNA. There were insufficient quantities of these materials, however, for further characterization.

In contrast to the above results, we have found that with an S9 preparation from phenobarbital-induced B6 mouse liver, the profile of [^{3}H]BP—DNA adducts in *S. typhimurium* did not resemble that seen with mammalian cells exposed to radioactive BP. This is consistent with previous studies comparing the [^{3}H]BP—DNA adducts formed in subcellular systems with various microsome preparations and calf-thymus DNA [4]. Although more definitive characterization of the products is required, it appears that with phenobarbital-induced S9 preparations there are significant amounts of BP-4,5-oxide-type DNA adducts, and lesser or negligible amounts of BPDE adducts; whereas, with Aroclor- and 3MC-induced S9 preparations, the BPDE adducts predominate. These differences presumably relate to the fact that the type of inducer used markedly influences the type of cytochrome p450 which is induced [6]. In separate mutagenesis assays with *S. typhimurium* TA98 we have found that the number of revertants per plate with the Aroclor-induced B6 mouse enzyme was approximately 5-fold greater than with the phenobarbital-induced enzymes. This is qualitatively consistent with the greater extent of [^{3}H]BP binding obtained with the former enzyme (Table 1), although a precise quantitative com-

parison is not possible because of the differences in reaction conditions used in the mutagenesis and DNA-binding studies. In the present study, the exposure of *S. typhimurium* to BP was done under the preincubation variation of the standard Ames assay [20]. We are not certain whether the same adducts would be formed if the exposure was done only in agar. The latter method could not be assessed because of the difficulties in obtaining sufficient DNA.

Concerning the radioactivity in the early regions of the LH-20 columns (fractions 5—20, Figs. 1A, B, C and 3A) it seems that at most 20% can be attributed to incomplete hydrolysis of the modified DNA. Most of this radioactivity is probably due to tritium exchange between [^{3}H]BP and unmodified bases, as was shown by Baird and Brookes [3] when [^{3}H]-7-methylbenz[*a*]anthracene was incubated with DNA in an in vitro microsomal system. The formation of this material is not restricted to the *S. typhimurium* system since it has also been seen when [^{3}H]BP and microsomes are incubated with DNA in vitro [4,11,17, 18]. This material is not detected, however, in hydrolysates of DNA obtained from human or bovine bronchial explants [8,19], hamster embryo-cell cultures [7] or 10T 1/2 cell cultures [5] previously exposed to [^{3}H]BP. It appears that the formation of this material is an artifact resulting from the use of disrupted microsomal enzyme preparations. Whatever the nature of this material, it is unlikely that it plays a significant role in the mechanism of BP mutagenesis in *S. typhimurium*. Although approximately equal amounts of this material are found in DNA samples obtained from experiments in which Aroclor- and phenobarbital-induced enzymes were used, the number of revertants with the Aroclor-enzyme system was 5-fold more than that obtained with the phenobarbital enzyme (R. Santella, D. Grunberger and I.B. Weinstein, unpublished studies).

Our finding that with the appropriate S9 enzyme preparation, the DNA of *S. typhimurium* contains a BPDE I—deoxyguanosine adduct which is identical to that found in several mammalian systems, including human bronchus, lends further justification to the use of the microsome-mediated *S. typhimurium* mutagenesis assay as an in vitro system for predicting potential human mutagens and carcinogens. The results do show, however, that differences in BP nucleoside adduct profiles can occur with various methods of preparing the S9-microsome system. Therefore, the arbitrary use of specific microsomal enzyme preparations to mimic the activation of diverse classes of agents by intact mammalian systems should be approached with caution.

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